# Activation and detoxification metabolism of urban air pollutants 2-nitrobenzanthrone and carcinogenic 3-nitrobenzanthrone by rat and mouse hepatic microsomes

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Running headline: Metabolism of 2-nitrobenzanthrone and 3-nitrobenzanthrone by hepatic microsomes

**OBJECTIVES:** 2-Nitrobenzanthrone (2-NBA) has recently been detected in ambient air particulate matter. Its isomer 3-nitrobenzanthrone (3-NBA) is a potent mutagen and suspected human carcinogen identified in diesel exhaust. Understanding which enzymes are involved in metabolism of these toxicants is important in the assessment of individual susceptibility. Here, metabolism of 2-NBA and 3-NBA by rat and mouse hepatic microsomes containing cytochromes P450 (CYPs), their reductase (NADPH:CYP reductase), and NADH:cytochrome $b_5$ reductase was investigated under anaerobic and aerobic conditions. In addition, using the same microsomal systems, 2-NBA and 3-NBA were evaluated to be enzymatically activated under anaerobic conditions to species generating 2-NBA- and 3-NBA-derived DNA adducts.

**METHODS:** High performance liquid chromatography (HPLC) with ultraviolet (UV) detection was employed for the separation and characterization of 2-NBA and 3-NBA metabolites formed by hepatic microsomes of rats and mice under the anaerobic and aerobic conditions. Microsomal systems isolated from the liver of the control (untreated) rats and rats pretreated with Sudan I, β-naphthoflavone (β-NF), phenobarbital (PB), ethanol and pregnenolon 16α-carbonitrile (PCN), the inducers of cytochromes P450 (CYP) 1A1, 1A1/2, 2B, 2E1 and 3A, respectively, were used in this study. Microsomes of mouse models, a control mouse line (wild-type, WT) and Hepatic Cytochrome P450 Reductase Null (HRN) mice with deleted gene of NADPH:CYP reductase in the liver, thus absenting this enzyme in their livers, were

also employed. To detect and quantify the 2-NBA- and 3-NBA-derived DNA adducts, the $^{32}P$ postlabeling technique was used.

**RESULTS:** Both reductive metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA), found to be formed predominantly under the anaerobic conditions, and two 3-NBA oxidative metabolites, whose structures have not yet been investigated, were formed by several microsomal systems used in the study. Whereas a 3-NBA reductive metabolite, 3-ABA, was found only in the microsomal systems of control rats, the rats treated with β-NF and PB, and microsomes of WT and HRN mice, all hepatic microsomes tested in the study were capable of activating this carcinogen under the reductive conditions to form DNA adducts. A stability of a reactive intermediate of 3-NBA, *N*-hydroxy-3-aminobenzanthrone that is formed during 3-NBA reduction to 3-ABA, to form nitrenium (and/or carbenium) ions binding to DNA in individual microsomes as well as binding of these ions to proteins of these microsomes, might be the reasons explaining this phenomenon. In contrast to 3-NBA, its isomer 2-NBA was not metabolized by any of the used enzymatic systems both under the anaerobic and aerobic conditions. Likewise, no DNA adducts were detectable after reaction of 2-NBA in these systems with DNA.

**CONCLUSIONS:** The results found in this study, the first report on the metabolism of 2-NBA and 3-NBA by rat and mouse hepatic microsomes demonstrate that 3-NBA, in contrast to 2-NBA, is reductively activated to form 3-NBA-derived DNA adducts by these enzymatic systems. NADPH:CYP reductase can be responsible for formation of these DNA adducts in rat livers, while NADH:cytochrome $b_5$ reductase can contribute to this process in livers of HRN mice.

**KEY WORDS**

74 2-nitrobenzanthrone; 3-nitrobenzanthrone, cytochrome P450; NADPH:cytochrome
75 P450 reductase; activation and detoxification metabolism.
76
77 **ABBREVIATIONS & UNITS**
78 3-ABA – 3-aminobenzanthrone
79 2-ABA – 2-aminobenzanthrone
80 β-NF – β-naphthoflavone
81 CYP - cytochrome P450
82 dA-$N^6$-3-ABA - 2-(2'-deoxyadenosin-$N^6$-yl)-3-aminobenzanthrone
83 dG-$N^2$-3-ABA - *N*-(2'-deoxyguanosin-$N^2$-yl)-3-aminobenzanthrone
84 dG-C8-*N*-3-ABA - *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone
85 DMSO - dimethyl sulfoxide
86 HPLC – high performance liquid chromatography
87 HRN mice - Hepatic cytochrome P450 reductase null mice
88 *N*-OH-2-ABA - *N*-hydroxy-2-aminobenzanthrone
89 *N*-OH-3-ABA - *N*-hydroxy-3-aminobenzanthrone
90 NADPH - nicotinamidadeninedinucleotide phosphate (reduced)
91 2-NBA – 2-nitrobenzanthrone
92 3-NBA – 3-nitrobenzanthrone
93 NQO1 - NAD(P)H:quinone oxidoreductase 1
94 PB – phenobarbital
95 PCN - pregnenolon 16α-carbonitrile
96 r.t. – retention time
97 RAL - relative adduct labeling
98 TLC - thin-layer chromatography

UV – ultraviolet

WT – wild-type

## INTRODUCTION

The increased lung cancer risk after exposure to the environmental pollutants nitro-polycyclic aromatic hydrocarbons and their detection in the lungs of non-smokers with lung cancer has led to considerable interest in assessing their potential cancer risk (IARC, 1989; Vineis and Husgafvel-Pursiainen, 2005).

The nitroaromatic 3-nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA, Figure 1) occurs in diesel exhaust and in airborne particulate matter (Enya et al., 1997, Seidel et al., 2002, Arlt, 2005, Nagy et al., 2005). 3-NBA exhibits extremely high mutagenic activity (Enya et al., 1997, Arlt, 2005) and is also a genotoxic carcinogen causing lung tumors in rats (Enya et al., 1997, Nagy et al., 2005). The predominant DNA adducts formed by 3-NBA after its metabolic activation by reduction of the nitro group (Arlt et al., 2003, 2005, Stiborova et al., 2006, 2008) are 2-(2'-deoxyguanosin-$N^2$-yl)-3-aminobenzanthrone and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (Arlt et al. 2004a, 2004b, 2006, Bieler et al., 2005; Vom Brocke, et al., 2009) and these are most probably responsible for the G to T transversion mutations induced by 3-NBA in Muta Mouse (Arlt et al., 2004c) and in *TP53* using human *TP53* knock-in (Hupki) murine embryonic fibroblasts (Vom Brocke, et al., 2009). The metabolic activation of 3-NBA is mediated primarily by cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1), while *N*,*O*-acetyltransferases (NATs) and sulfotransferases (SULTs) are the major activating phase II enzymes (Arlt et al., 2005, Stiborova et al., 2006, 2008) (Figure 1).

2-Nitrobenzanthrone (2-NBA), an isomer of 3-NBA, has been detected in ambient air particulate matter (Phousongphouang and Arey, 2003). Using a semiempirical quantum mechanical approach recent studies indicated small differences between 2-NBA and 3-NBA in the reduction potential and the geometry of the nitro group attached to the benzanthrone skeleton (Takamura-Enya et al., 2006, Arlt et al., 2007; Reynisson et al., 2008, Nagy et al., 2007). Only differences in hydrophobicity were observed between these NBA isomers, indicating that penetration through cell membranes seems not to be identical for both compounds (Takamura-Enya et al., 2006, Arlt et al., 2007, Stiborova et al., 2010b). The mutagenic and genotoxic potential of 2-NBA is, however, much lower than that of 3-NBA (Takamura-Enya et al., 2006, Arlt et al., 2007). Although 2-NBA has been shown to be genotoxic *in vitro* (Arlt et al., 2007, Nagy et al., 2007), lack of genotoxicity *in vivo* in rats (*e.g.* DNA adduct formation) was reported (Arlt et al., 2007). Nevertheless, its higher abundance than 3-NBA in ambient air urges further investigation to assess its potential hazard to human health.

Recently, we have compared the efficiencies of human enzymatic systems such as hepatic cytosols and microsomes, NAD(P)H:quinone oxidoreductase 1 (NQO1), xanthine oxidase, NADPH:CYP reductase, *N*,*O*-acetyltransferases and sulfotransferases) and human primary hepatocytes to activate 2-NBA and its isomer 3-NBA to species forming DNA adducts (Stiborova et al., 2010b). We have found that in contrast to 3-NBA, 2-NBA was not metabolized at detectable levels by the tested human enzymatic systems and enzymes expressed in human hepatocytes and no DNA adducts were generated by 2-NBA. Therefore, we have also concluded that 2-NBA seems to possess a relatively lower risk to humans than 3-NBA (Stiborova et al., 2010b).

148 The aim of the present study was to investigate whether both studied
149 environmental pollutants can be metabolized by other enzymatic systems, namely by
150 hepatic microsomes of two animal models, rat and mice. Both reductive metabolism
151 carried out under the anaerobic conditions and oxidative (aerobic) metabolism were
152 examined. In addition, using the same microsomal systems, 2-NBA and 3-NBA were
153 evaluated to be enzymatically activated under the anaerobic conditions to species
154 generating 2-NBA- and 3-NBA-derived DNA adducts.

155

156 **MATERIAL AND METHODS**

157 *Chemicals and enzymes.* Microsomes from rat livers were isolated and
158 characterized for CYP activities as described (Stiborova et al., 2010a, 2011).
159 NADPH, calf thymus DNA and dimethylsulfoxide (DMSO) were obtained from Sigma
160 Chemical Co (St Louis, MO, USA), Enzymes and chemicals for the $^{32}$P-postlabeling
161 assay were obtained from sources described (Arlt et al., 2004a, 2004b, 2004c,
162 Phillips, and Arlt, 2007). All these and other chemicals were reagent grade or better.

163 *Synthesis of 2-NBA, 3-NBA, 3-ABA and N-hydroxy-3-aminobenzanthrone (N-OH-*
164 *3-ABA).* 2-NBA was synthesized as reported (Suzuki et al., 1997). 3-NBA, 3-ABA and
165 *N*-OH-3-ABA were synthesized as described (Arlt et al., 2003, 2004b, Osborne e al.,
166 2005). Their authenticity was confirmed by UV spectroscopy, electrospray mass
167 spectrometry and high field $^{1}$H NMR spectroscopy. Their purity was analyzed using
168 HPLC, being >99.9% based on this method (Arlt et al., 2007).

169 *Preparation of microsomes and assays.* The study was conducted in accordance
170 with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry
171 of Agriculture, Czech Republic), which complies with the Declaration of Helsinki.
172 Microsomes from livers of ten male untreated Wistar rats and those of ten male rats

pre-treated with Sudan I, β-naphthoflavone (β-NF), phenobarbital (PB), ethanol and pregnenolone-16α-carbonitrile (PCN) were prepared by the procedure described previously (Stiborova et al., 2003, 2011, Naiman et al., 2010). Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with the bovine serum albumin as a standard (Weichelman et al., 1988). The concentration of CYP was estimated according to Omura and Sato (Omura and Sato, 1964) based on absorption of the complex of reduced CYP with carbon monoxide. Hepatic microsomes of control (uninduced) rats and rats induced with Sudan I, β-NF, phenolbarbital (PB), ethanol and PCN contained 0.6, 1.8, 1.3, 2.7, 1.8 and 1.6 nmol CYP/mg protein, respectively. The activity of NADPH:CYP reductase in rat hepatic microsomes was measured according to Sottocasa *et al*. (1967) using cytochrome c as substrate (i.e., as NADPH:cytochrome c reductase). NADPH:CYP reductase activities in hepatic microsomes of control (uninduced) rats and rats induced with Sudan I, β-NF, PB, ethanol and PCN were 0.210, 0.202, 0.199, 0.325, 0.201 and 0.290 μmol/min/mg protein, respectively.

Mouse hepatic microsomes from livers of four HRN (Hepatic Cytochrome P450 Reductase Null) (*Por*$^{lox/lox}$ + *Cre*$^{ALB}$) mice (Henderson et al., 2003) and four wild-type (WT) mice were isolated using the same procedures as described above. Hepatic microsomes of WT and HRN mice contained 0.3 and 0.4 nmol CYP/mg protein, respectively. NADPH:CYP reductase activity in hepatic microsomes of WT mice was 0.120 μmol/min/mg protein, but it was not detectable in microsomes of HRN mice.

*Incubations.* Incubation mixtures used for activation of 2-NBA and 3-NBA metabolism by rat and mouse hepatic microsomes to species forming DNA adducts were carried out as described previously (Arlt et al., 2003, Stiborova et al., 2010b). Briefly, the deaerated and argon-purged incubation mixtures, in a final volume of 750

198 µl, consisted of 50 mM Tris/HCl buffer (pH 7.4), 1 mM NADPH, rat or mouse hepatic
199 microsomes (1 mg of protein), 0.5 mg of calf thymus DNA and 100 µM 2-NBA or 3-
200 NBA (dissolved in 12.5 µl dimethyl sulfoxide - DMSO). This relatively high
201 concentration of DMSO in reaction mixtures, which might inhibit activities of some
202 CYPs, was necessary to be used, because of the very low solubility of 2-NBA or 3-
203 NBA. The same volume of DMSO was added into the control sample (without 2-NBA
204 or 3-NBA). In the case of mouse hepatic microsomes, incubation mixtures of the
205 same composition, but containing of 1 mM NADH instead of 1 mM NADPH, were
206 used. The reaction was initiated by adding 2-NBA or 3-NBA. Incubations were carried
207 out at 37°C for 2 hr; 3-NBA-derived DNA adduct formation was found to be linear up
208 to 3 hr (Arlt et al., 2003). Control incubations were carried out (*i*) without activating
209 system (microsomes), (*ii*) without NADPH, (iii) without DNA or (*iv*) without 3-NBA or
210 2-NBA. After the incubation and extraction with ethyl acetate, DNA was isolated from
211 the residual water phase by the phenol/chloroform extraction method as described
212 (Arlt et al., 2003).

213 Incubations mixtures used to study 2-NBA and 3-NBA metabolism by rat and
214 mouse hepatic microsomes, containing final volume of 500 µl, consisted of 50 mM
215 Tris/HCl buffer (pH 7.4), 1 mM NADPH, 0.5 mg of microsomal protein and 50 µM 2-
216 NBA or 3-NBA (dissolved in DMSO). The reaction was initiated by adding 2-NBA or
217 3-NBA. Reaction mixtures were incubated at 37°C for 60 min both under the aerobic
218 conditions (in open tubes) and under the anaerobic conditions that are described
219 above (see incubation conditions used to study activation of 2-NBA and 3-NBA to
220 form DNA adducts). In control incubations NADPH was omitted from the mixtures.
221 After incubation, the mixtures were extracted twice with ethyl acetate (2×1 ml) and 5
222 µl of 1 mM phenacetine in methanol was added as an internal standard. The extracts

were evaporated to dryness; residues were dissolved in 30 µl of methanol, and subjected to reverse phase-HPLC to evaluate the amounts of 2-aminobenzanthrone (2-ABA) or 3-ABA formed.

*HPLC.* HPLC was performed with a Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometric detector set at 254 nm, and peaks were integrated with a Chromeleon$^{TM}$ 6.01 integrator (Stiborova et al., 2010b; Klejdus et al., 2005). The column used was a Nucleosil 100-5 $C_{18}$ (Macherey-Nagel, Düren, Germany, 25 cm $\times$ 4.6 mm, 5 µm), preceded by a C-18 guard column. Chromatography was under isocratic conditions of 70% methanol in water, with a flow rate of 0.6 ml/min. Recoveries of 2-NBA, 3-NBA and 3-ABA were around 95%. 3-ABA, 3-NBA and 2-NBA were eluted with retention times (r.t.) of 7.8, 24.6 and 25.4 minutes, respectively (Figure 2)

*$^{32}$P-Postlabeling analysis of DNA adducts*. $^{32}$P-Postlabeling analysis using n-butanol extraction, and thin layer chromatography (TLC) and HPLC were performed as described (Arlt et al, 2006, Stiborova et al., 2010b). Enrichment by *n*-butanol extraction has been shown to yield more adduct spots and a better recovery of 3-NBA-derived DNA adducts than enrichment by nuclease P1 digestion (Arlt et al, 2004a, 2004b, 2004c, 2006, Stiborova et al., 2006). The detection limit certain individual adducts was around 1 adduct per $10^{10}$ nucleotides. 3-NBA-derived DNA adducts were identified using standards of 2-(2'-deoxyadenosin-*N*$^6$-yl)-3-aminobenzanthrone-3'-phosphate, *N*-(2'-deoxyguanosin-*N*$^2$-yl)-3-aminobenzanthrone-3'-phosphate and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone-3'-phosphate as described (Arlt et al, 2006, Stiborova et al., 2010b).

## RESULTS AND DISCUSSION

*Metabolism of 2-NBA and 3-NBA by rat and mouse hepatic microsomes.* In initial experiments, 3-NBA was incubated with rat hepatic microsomes in the presence of NADPH, a cofactor of CYP- and NADPH:CYP reductase-mediated reactions, under the anaerobic conditions. Using HPLC with UV detection, one major 3-NBA reduction product (see Figure 2) was found. Using co-chromatography with synthetic standards, this peak was identified to be the 3-NBA reduction product, namely 3-ABA (Figure 2). No 3-ABA was detectable when NADPH was omitted from the incubation mixtures. This finding suggest that either NADPH:CYP reductase or CYP enzymes might be responsible for reduction of 3-NBA to 3-ABA. A time-dependent decrease in 3-NBA in incubation mixture corresponded to an increase in 3-ABA formation (data not shown). No *N*-OH-3-ABA was detectable by HPLC, although it should be formed during 3-NBA reduction to 3-ABA and as a reactive intermediate binding to DNA (Arlt et al., 2003, 2005). Because of the reactivity of this intermediate, it is easily converted to the nitrenium and/or carbenium ion (Figure 1), which may be scavenged by the microsomal proteins present in the incubation mixtures.

In the case of a rat animal model, besides hepatic microsomes of control (untreated) rats, we have also used hepatic microsomes of rats treated with several CYP inducers such as Sudan I and β-NF, which are inducers of CYP1A1/2, PB, which is an inducer of CYP2B, ethanol, which increases the levels of CYP2E1 and PCN, which induces CYP3A. Under the anaerobic conditions, hepatic microsomes of control (untreated) rats and microsomes of rats treated with β-NF and PB were capable of reducing 3-NBA to 3-ABA, having almost the same efficiencies to form this 3-NBA metabolite (Table 1). Surprisingly, hepatic microsomes of rats treated with β-NF reduced 3-NBA even under the aerobic conditions (Table 1). This unexpected

result remains to be explained. In contrast to these results, the final reduction metabolite of 3-NBA, 3-ABA, was not detectable in other microsomes tested in this study (Table 1). Amounts of 3-ABA formed by individual rat hepatic microsomes did not correspond either to specific contents of CYP or to activities of NADPH:CYP reductase in these microsomes. This finding suggests that amounts of 3-ABA detectable in microsomal systems does not depend only on the activities of both types of enzymes, but also on other, still unknown, factors. A stability of a reactive intermediate of 3-NBA, *N*-OH-3-ABA that is formed during 3-NBA reduction to 3-ABA to form nitrenium (and/or carbenium) ions binding to DNA in individual microsomes, as well as binding of these ions to microsomal proteins, might be the reasons explaining this phenomenon. These results also indicate that the determination of amounts of the final reductive metabolite of 3-NBA, 3-ABA, seems not to be a suitable marker which should be used to evaluate efficiencies of enzymatic systems to reduce 3-NBA, the crucial reaction leading to 3-NBA-derived DNA adducts (Figure 1).

Hepatic microsomes of WT and HRN mice were also able to reduce 3-NBA to 3-ABA, both under the anaerobic and aerobic conditions. Lower amounts of 3-ABA was formed by hepatic microsomes of HRN mice, but these microsomes were more effective in this reaction under the aerobic than the anaerobic conditions (Table 1).

Under the aerobic conditions, hepatic microsomes of control rats and rats pretreated with PB, ethanol and PCN were also capable of oxidizing 3-NBA. Using HPLC with UV detection, up to two 3-NBA oxidation products, eluted with the retention times (r.t.) of 13 and 18 min, were found (data not shown). Hepatic microsomes of control rats generated both these metabolites, whereas microsomes of rats treated with PB formed only the metabolite eluting at 13 min and microsomes

298 of rats pretreated with ethanol and PCN formed the metabolite eluting at 18 min.
299 Structures of these metabolites remain to be characterized. No such metabolites
300 were generated by hepatic microsomes of the rats treated with other CYP inducers
301 and microsomes of both mouse models.

302 In contrast to 3-NBA that is reduced by several microsomal systems to 3-ABA,
303 no 2-aminobenzanthrone (2-ABA) was found to be generated from 2-NBA by all
304 hepatic microsomes used in the experiments (see Figure 2). Likewise, no oxidation
305 product peaks were detectable by HPLC employed to separate the 2-NBA
306 metabolites.

307 *Activation of 2-NBA and 3-NBA to species forming DNA adducts.* In initial
308 experiments, we compared the DNA adduct formation by 2-NBA and 3-NBA using
309 hepatic microsomal systems under standardized experimental conditions, in the
310 presence of NADPH, a cofactor of microsomal enzymes (NADPH:CYP reductase
311 and CYPs). The same rat and mouse hepatic microsomes that were used to study
312 metabolism of 2-NBA and 3-NBA were utilized. The formation of DNA adducts was
313 analyzed using the butanol extraction version of the $^{32}$P-postlabeling method.

314 In accordance with our previous *in-vitro* studies (Bieler et al., 1999, Arlt et al.,
315 2003, 2005, Stiborova et al., 2006, 2008), the DNA adduct pattern generated from 3-
316 NBA by these enzymatic systems, consisted of a cluster of at least five adducts
317 (spots indicated with arrows in Figure 3) essentially identical to those found
318 previously *in vivo* in rats and mice treated with this carcinogen (Arlt et al., 2003,
319 2005, Bieler et al., 2005, 2007). Co-chromatographic analysis of individual spots on
320 HPLC confirmed that adduct spots are derived from 3-NBA by nitroreduction (data
321 not shown). Three of these adducts were identified as 2-(2'-deoxyadenosin-$N^6$-yl)-3-
322 aminobenzanthrone (dA-$N^6$-3-ABA, spot 1), 2-(2'-deoxyguanosin-$N^2$-yl)-3-

aminobenzanthrone (dG-$N^2$-3-ABA, spot 3) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-3-ABA, spots 4,5) (see Fig. 3). TLC autoradiograms of $^{32}$P-labeled DNA from control incubations carried out in parallel without activation systems, without DNA, or without 3-NBA or without NADPH, a cofactor of microsomal enzymes (CYPs and NADPH:CYP reductase), were devoid of adduct spots in the region of interest (data not shown). These results indicate that both CYPs and NADPH:CYP reductase might be responsible for the reductive activation of 3-NBA leading to the formation of these DNA adducts.

In the species comparison, microsomes from rats were more efficient to activate 3-NBA than those of mice, mainly the microsomes of HRN mice. The efficiency of rat and mouse hepatic microsomes to activate 3-NBA corresponded to the NADPH:CYP reductase activity (Table 2). Incubations of 3-NBA and DNA with hepatic microsomes of rats pretreated with inducers of NADPH:CYP reductase, PCN and PB, led to a 4- and 3-fold increase, respectively, in the formation of 3-NBA-DNA adducts, while lower activities of this enzyme in mouse hepatic microsomes resulted in a decrease in levels of 3-NBA-DNA adducts (Table 2). These results suggest that NADPH:CYP reductase might be the major enzyme responsible for reductive activation of 3-NBA to form DNA adducts in hepatic microsomes (Arlt et al., 2003). Interestingly, 3-NBA-derived DNA adducts were also formed in hepatic microsomes of HRN mice, where no activity of NADPH:CYP reductase was detectable. Therefore, another enzyme should be responsible for 3-NBA activation in these microsomes. Using NADH, a cofactor of NADH:cytochrome $b_5$ reductase in incubation mixtures containing hepatic microsomes of HRN mice, higher levels of 3-NBA-derived DNA adducts were found (Table 3). Hence, NADH:cytochrome $b_5$ reductase can substitute the NADPH:CYP reductase in reductive activation of 3-NBA in hepatic microsomes of HRN mice.

In contrast to the results found with 3-NBA, no 2-NBA-derived DNA adducts were detected under the same experimental conditions with any of the hepatic microsomes used in the experiments (Figure 2). This finding corresponds fully with the lack of 2-NBA reduction in these hepatic microsomal enzymatic systems (see Figure 2).

## CONCLUSIONS

The results of this study show that in contrast to 3-NBA, 2-NBA is not a substrate of the enzymes present in the rat and mouse hepatic microsomal systems tested in our experiments. Using the $^{32}$P-postlabeling assay, which is in principle sensitive enough to detect DNA adduct levels as low as 1 adduct per $10^{10}$ normal nucleotides (Phillips and Arlt, 2007), we were unable to detect 2-NBA-derived DNA adducts catalyzed by any of the microsomal enzymatic systems utilized. Our results correspond to data published previously showing that 2-NBA-derived DNA adducts were not detected both *in vitro*, in several human enzymatic systems (Stiborova et al, 2010b) and in Wistar rats treated with 2-NBA *in vivo* (Arlt et al., 2007). Only after the administration of the reactive *N*-hydroxylated intermediate *N*-hydroxy-2-aminobenzanthrone (*N*-OH-2-ABA), 2-NBA-derived DNA adducts were detectable in several rat organs such as lung, liver, kidney, colon and pancreas (Arlt et al., 2007).

In the case of 3-NBA, this carcinogen is reduced by rat and mouse hepatic microsomes to species forming 3-NBA-derived DNA adducts. NADPH:CYP reductase seems to be the major enzyme reductively activating 3-NBA in rat and/or mouse liver microsomes. However, when this enzyme is absenting in the microsomes, like it is in hepatic microsomes of HRN mice, NADH:cytochrome $b_5$ can substitute NADPH:CYP reductase in this activation reaction. The results found in the experiments determining the amounts of the final reductive metabolite of 3-NBA, 3-

ABA, and levels of 3-NBA-derived DNA adducts strongly suggest that determination of the levels of these DNA adducts are a more suitable method to evaluate the enzymes responsible for reductive activation of this carcinogen than that of amounts of formed 3-ABA.

Finally, the present study has increased our knowledge on the potential of rat and mouse hepatic microsomal enzymatic systems to reductively activate the two NBA urban air pollutants, 2-NBA and 3-NBA. In accordance with previous data our results indicate that 3-NBA is a potent genotoxin and carcinogen (Arlt et al., 2003, 2005, Bieler et al., 2007, Stiborova et al., 2006, 2008, 2010b), supporting our efforts in investigating the mechanisms of 3-NBA carcinogenicity.

**ACKNOWLEDGMENT** Supported by the Grant Agency of the Czech Republic (grants 503/10/0136) and Charles University in Prague (UNCE 204025/2012).

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518

519 Table 1. Amounts of 3-ABA formed from 3-NBA during its incubation with different rat
520 or mouse hepatic microsomes
521

| Hepatic microsomal system of | Experimental conditions | 3-ABA (μmol/mg protein) |
|---|---|---|
| control rats | Anaerobic | 5.2 ± 0.1 |
| | Aerobic | 0 |
| rats treated with Sudan I | Anaerobic | 0 |
| | Aerobic | 0 |
| rats treated with β-NF | Anaerobic | 4.6 ± 0.5 |
| | Aerobic | 4.6 ± 0.1 |
| rats treated with PB | Anaerobic | 5.8 ± 0.9 |
| | Aerobic | 0 |
| rats treated with ethanol | Anaerobic | 0 |
| | Aerobic | 0 |
| rats treated with PCN | Anaerobic | 0 |
| | Aerobic | 0 |
| control mice (WT mice) | Anaerobic | 3.1 ± 0.8 |
| | Aerobic | 3.6 ± 0.3 |
| HRN mice | Anaerobic | 1.5 ± 0.2 |
| | Aerobic | 2.8 ± 0.3 |

522
523 Experimental conditions are described in Material and methods. NADPH was used in
524 all incubations mixtures. Values in the table are averages and standard deviations of
525 three determinations.
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530 Table 2. Total levels of DNA adducts formed after activation of 3-NBA by different rat
531 or mouse hepatic microsomes in the presence of NADPH
532

| Hepatic microsomal system of | Experimental conditions | Total levels of 3-NBA-DNA adducts (RAL/$10^8$ nucleotides) |
|---|---|---|
| control rats | Anaerobic | 4.2 ± 0.4 |
| rats treated with β-NF | Anaerobic | 3.4 ± 0.3 |
| rats treated with PB | Anaerobic | 12.1 ± 1.0 |
| rats treated with ethanol | Anaerobic | 3.9 ± 0.4 |
| rats treated with PCN | Anaerobic | 15.9 ± 1.3 |
| control mice (WT mice) | Anaerobic | 0.66 ± 0.05 |
| HRN mice | Anaerobic | 0.54 ± 0.05 |

533
534 Experimental conditions are described in Material and methods. 1 mM NADPH was
535 used in all incubations mixtures. Values in the table are averages and standard
536 deviations of three determinations. RAL – relative adduct labeling.
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542 Table 3. Total levels of DNA adducts formed after activation of 3-NBA by mouse
543 hepatic microsomes in the presence of NADH
544

| Hepatic microsomal system of | Experimental conditions | Total levels of 3-NBA-DNA adducts (RAL/$10^8$ nucleotides) |
|---|---|---|
| control mice (WT mice) | Anaerobic | 0.25 ± 0.03 |
| HRN mice | Anaerobic | 0.91 ± 0.08 |

545
546 Experimental conditions are described in Material and methods. 1 mM NADH was
547 used in incubations mixtures. Values in the table are averages and standard
548 deviations of three determinations. RAL – relative adduct labeling.
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Figure 1. Pathways of metabolic activation and DNA adduct formation of 3-nitrobenzanthrone and 3-aminobenzanthrone. See text for details. 3-NBA, 3-nitrobenzanthrone; 3-ABA, 3-aminobenzanthrone; NQO1, NAD(P)H:quinone oxidoreductase; NAT, *N*,*O*-acetyltransferases; SULT, sulfotransferase; CYP, cytochrome P450; HRP, horseradish peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; PHS-1, prostaglandin H synthase 1 (cyclooxygenase 1); POR, NADPH:CYP oxidoreductase; R = $-COCH_3$ or $-SO_3H$; dA-$N^6$-ABA, 2-(2'-deoxyadenosin-$N^6$-yl)-3-aminobenzanthrone; dG-$N^2$-ABA, *N*-(2'-deoxyguanosin-$N^2$-yl)-3-aminobenzanthrone; dG-C8-*N*-ABA, *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone.

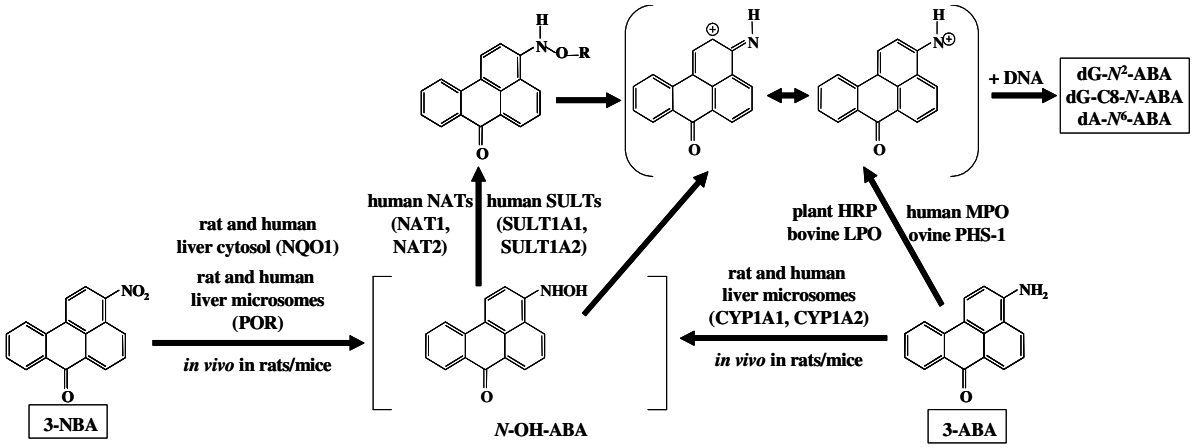

Figure 2. HPLC chromatographs of 2-NBA (A) and 3-NBA (B) metabolites produced by human NQO1 under the anaerobic conditions. Experimental conditions are described in Material and methods.

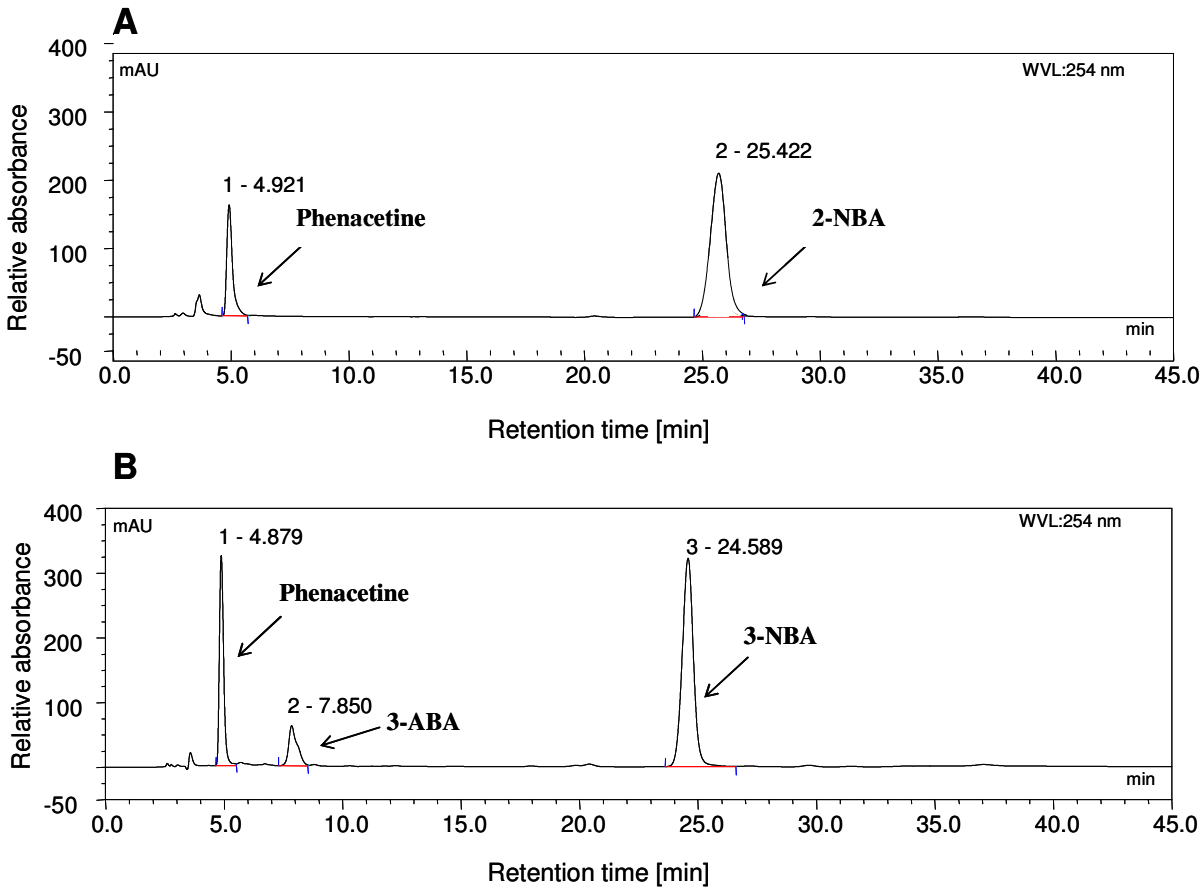

Figure 3. Autoradiographic profiles of DNA adducts generated by 100 μM 2-NBA (A) and 100 μM 3-NBA (B) after activation with hepatic microsomes of rats pretreated with PCN. Experimental conditions are described in Material and methods. Spot 1, dA-$N^6$-3-ABA, spot 3, dG-$N^2$-3-ABA, and spots 4,5, dG-C8-*N*-3-ABA.